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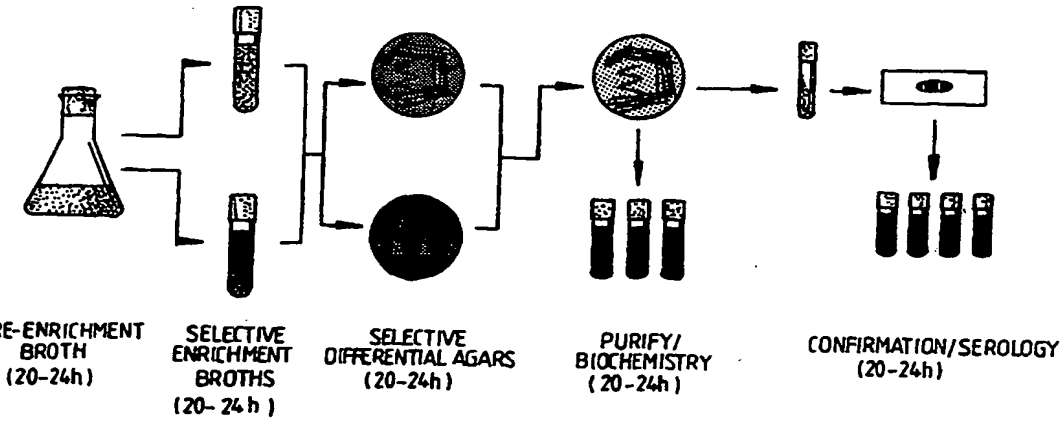

(57) Abstract

A method of enriching the population of a target microorganism in a sample, comprises incubating the sample in a pre–enrichment medium, with one or more selective agents to favour growth of the target microorganism arranged for release into the medium after a predetermined time delay. The method of the invention combines pre–enrichment and selective enrichment steps by use of timed release of the selective agent(s). This has the benefits of reducing the amount of manipulation and labour necessary for these stages, and also of reducing the amount of time required for these stages. It has been found that these stages can be satisfactorily completed within 24 hours, without the need for operator intervention. The enrichment can be followed by identification of the target microorganism, using any appropriate technique, either conventional or rapid. Using rapid identification techniques, total test time can be reduced to a little over 24 hours. The invention finds particular application in the analysis of samples of foodstuffs and beverages in the enrichment and identification of a wide range of foodborne pathogens including *Salmonella, Listeria, Campylobacter* and *E. coli* 0157, but can also be used in connection with a wider range of target organisms from a much wider range of sample types including environmental samples.

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Title: SELECTIVE ENRICHMENT AND DETECTION OF MICROORGANISMS

Field of the Invention

This invention relates to the detection of microorganisms, for example in food samples, and particularly concerns a method of enriching the population of a target microorganism in a sample, and ingredients for use in such a method.

Background to the Invention

For public safety and quality control purposes the analysis of samples of consumer products, such as foodstuffs and beverages, for the presence of pathogenic microorganisms is conducted on an extremely wide scale. Because pathogenic microorganisms such as *Listeria* and *Salmonella* are capable of proliferating very rapidly under the right conditions, the presence of even a single viable cell of such organisms in a foodstuff may give rise to serious infection after the foodstuff has been ingested by a human. It is therefore necessary for such organisms to be detectable even when present at extremely low levels.

A typical conventional technique for detecting a target microorganism of interest, eg *Salmonella*, involves incubating a sample in a pre-enrichment broth, commonly buffered peptone water (BPW) for 18 to 24 hours under conditions which encourage microorganism recovery and growth, so that any organisms present can proliferate in the sample and attain population levels which are more readily detectable. This step is known as a pre-enrichment, recovery or resuscitation step.

Portions of the pre-enrichment culture are then subcultured into selective enrichment broths and incubated for a further 20 to 24 hours. The selective enrichment broths are designed to inhibit growth of comparatively innocuous non-target microorganisms and so

favour the growth of the target microorganisms. This step is known as a selective enrichment step.

Figure 1 is a simplified schematic graph illustrating the variation in cell numbers with time during the pre-enrichment step (0 to 24 hours) and the enrichment step (24 to 48 hours) of a target organism (*Salmonella)* and non-target, competitor organisms.

After the enrichment step, the target organisms are then identified. Conventional processing involves subculturing enrichment broths onto selective differential agar plates and incubating for 20 to 24 hours. Suspected colonies of target organism are identified by visual examination, and selected suspected colonies are removed from the plates, purified and identified, eg using triple sugar iron (TSI) agar and lysine iron (LSI) agar slopes and serological tests. Purification, identification and confirmation can take up to 48 hours.

Figure 2 is a schematic representation of such a conventional method for detection of *Salmonella* in foods.

Rapid methods of identifying microorganisms, eg using ELISA, electrical based methods, nucleic acid probes, PCR etc, have been devised and can give results in as little as 30 minutes. Despite promising results in laboratory-based experiments, when used in real food enrichments even the most sensitive commercially available systems require at least 10^4 target cells ml^{-1} of enrichment broth to generate a reproducible result. This requires that pre-enrichment and selective enrichment are still used in order to achieve this number of target cells. Many of the new rapid tests have an even lower degree of sensitivity which can often be further reduced by high numbers of competing microorganisms.

In order for total test times to become shorter, detection systems either have to become more sensitive or enrichment culture has to be made more effective. It is likely that detection systems will become more sensitive, but problems associated with sample matrix interference and detection of material from non-viable organisms will still have to be resolved. In the meantime, there is scope for time savings to be made in the area of pre-

enrichment and selective enrichment broth culture which currently take two days to perform.

Not surprisingly, there have been numerous attempts to shorten pre-enrichment but few appear successful. For example, D'Aoust and Maishment in Journal of Food Protection 42, 153-157 (1979) reported that pre-enrichment for 6 hours in various non-selective media failed to identify about half of low and high moisture foods contaminated with *Salmonella*. Subsequently, D'Aoust *et al* in Journal of Food Protection 53, 562-565 (1990) reported that shorter incubation times, 3-8 hours, in non-selective enrichment media did not result in effective resuscitation of stressed organisms, and gave unacceptably high numbers of false negative results. Such research illustrates that there is much to learn about the complex processes and interactions that take place during the primary stage of the *Salmonella* isolation method.

Pre-enrichment is designed to perform many functions, including maintenance of neutral pH, both against the effect of the food material and the acid produced by growth of competing bacteria, and ensuring that the level of growth of the target species is sufficient to guarantee transfer and growth upon subsequent subculture to the selective enrichment broth. The most important role of pre-enrichment, however, is in supporting the resuscitation of cells of the target microorganism that maybe in a debilitated state. Without some provision to rejuvenate such stressed or damaged cells, the selective conditions required in the culture isolation procedure could render these bacteria undetectable. Such an occurrence could have serious consequences since even low levels of organisms such as *Salmonella* have been known to cause infection.

Summary of the Invention

In one aspect, the present invention concerns a method of enriching the population of a target microorganism in a sample, comprising incubating the sample in a pre-enrichment medium, with one or more selective agents to favour growth of the target microorganism arranged for release into the medium after a predetermined time delay.

The method of the invention combines pre-enrichment and selective enrichment steps by use of timed release of the selective agent(s). The selective agents(s) are released in automated manner, in a way that does not require operator intervention. This has the benefits of reducing the amount of manipulation and labour necessary for these stages, and also of reducing the amount of time required for these stages. It has been found that these stages can be satisfactorily completed within 24 hours, without the need for operator intervention.

The enrichment can be followed by identification of the target microorganism, using any appropriate technique, either conventional or rapid, as outlined above. Using rapid identification techniques, total test time can be reduced to a little over 24 hours, and further time reductions may become possible.

The invention finds particular application in the analysis of samples of foodstuffs and beverages in the enrichment and identification of a wide range of foodborne pathogens including *Salmonella, Listeria, Campylobacter* and *E. coli* O157, but can also be used in connection with a wider range of target organisms from a much wider range of sample types, including environmental samples.

The pre-enrichment medium conveniently comprises peptone. The peptone is preferably selected for optimised recovery of stressed or damaged cells of the target microorganism. The peptone is preferably generally as described in the specification of our copending British Patent Application No. 9721396.1 (pursued in a PCT application).

The pre-enrichment medium desirably includes one or more recovery agents to aid recovery of stressed or damaged cells of the target microorganism. The currently preferred recovery agent is OXYRASE enzyme, which is made from sterilised bacterial membrane fragments, and which is known to be an effective oxygen-reducing enzyme used to produce anaerobic conditions. The OXYRASE enzyme is described in technical bulletins distributed by Oxyrase, Inc. of Ohio and is further described by Adler et al. in J. Bacteriology, August 1981, 326-332, and in a paper by H.I Adler in Critical Reviews of Biotechnology 10:118 (1990) entitled "The Use of Microbial Membranes to Achieve

Anaerobiosis". See also US Patents Nos. 4476224, 4996073 and 5204853 and WO88/04319. The word OXYRASE is a trade mark of Oxyrase, Inc, from whom OXYRASE enzyme is available. A similar enzyme system derived from irradiated microorganisms is described in EP 0520757 of Becton Dickinson.

In order to achieve complete recovery of all stressed target cells, before the Jameson Effect occurs and growth of non-target cells inhibits growth of target cells, it is necessary to optimise the selection of peptone and concentration of recovery agent. See, for example, Jameson, *J. Hyg. Cambs.* (1962) Vol. 60 p193-207 for a discussion of the Jameson Effect. Poorly performing peptones combined with high concentrations of recovery agent such as OXYRASE, and optimised peptones combined with lower concentrations of OXYRASE, will achieve similar results in terms of speed and extent of recovery. OXYRASE is expensive so it is therefore beneficial to use it at the lowest concentration possible that will still support maximum recovery. For this reason, use of an optimised peptone is highly desirable.

It is believed that there are a number of ways in which oxidative stress is involved in pre-enrichment:

a) The better performing peptones are probably contributing the lowest level of toxic oxidising species, eg hydrogen peroxide, hydroxyl radicals, superoxide anions and singlet oxygen, to the medium, possibly generated by exposure to oxygen, light and/or high temperatures, eg on autoclaving.

b) Stressed cells are likely to be generating intracellular toxic oxidising species such as those mentioned above in a way that is independent of external levels. This explains why adding external catalase does not give maximum improvement to recovery. It is also possible that internal toxic oxidising species are generated at different rates in the different peptones.

c) OXYRASE causes the cells to grow anaerobically thus bypassing any internal pathways that produce toxic oxidising species such as hydrogen peroxide. OXYRASE also

contains catalase which removes external hydrogen peroxide which is why when it is added to poor peptones at high concentrations it is still effective at improving recovery. Additionally, free nucleic acid and lipid material in the OXYRASE preparation may absorb toxic oxidising species.

Anaerobic conditions can alternatively be achieved by physical means either using anaerobic jars, roll tubes (eg. the Hungate technique) or anaerobic cabinets. The latter two methods are likely to give similar improvements to use of recovery agents such as OXYRASE.

Sphingosine type selective agents may optionally be added as supplements to the pre-enrichment medium.

In some cases it may be appropriate to add other selective agents to the pre-enrichment medium, such as additional selective dyes and/or antibiotics.

When testing samples containing reducing agents in significant amounts, in particular sulphites and nitrites added as preservatives, it is found the reducing agents act to reduce the efficacy of recovery agents such as OXYRASE. This can be overcome by adding to samples containing or suspected to contain significant quantities of preservatives, such as sausages, neutralising agents such as pyruvate, alpha ketoglutaric acid, acetaldehyde, maltose, glucose, etc, which convert the reducing agent to a non-reactive form.

The selective agent(s) for delayed release may be chosen from a wide range of possibilities, having regard to the target organism, as is known to those skilled in the art. Good results have been obtained with selective agents based on the traditional Rappaport-Vassiliadis (RV) broth, including magnesium chloride and malachite green. Similar results may be expected using one or more other traditional selective agents such as dyes, selenite cystine, tetrathionate and antibiotics. Suitable concentrations of such materials are known to those skilled in the art, or can be readily determined by experiment.

Timed release of the selective agent(s) into the pre-enrichment medium is conveniently

achieved by incorporating the selective agent(s) in suitable timed-release capsules. The currently preferred capsule is generally as described in WO95/10263, and comprises an insoluble plastics capsule body with a male hydrogel plug. When the capsule is exposed to an aqueous medium the hydrogel plug swells slowly and eventually disengages, releasing the capsule contents, after a predetermined time interval that is controllable and predictable. Suitable capsules of this construction are available from Scherer DDS under the name PULSINCAP (PULSINCAP is a Trade Mark).

The time delay before release of the selective agents is chosen having regard to the target organism. It is necessary to try to achieve maximum recovery of target cells before overgrowth of non-target competitors.

Factors such as ingredients, time delay and incubation temperature are tailored to the particular target organism. For *Salmonella*, for example, good results have been obtained by incubation in pre-enrichment medium of optimised peptone with OXYRASE recovery agent, with Rappaport-Vassiliadis selective agents in size "O" PULSINCAP capsules designed to release their contents after 5 hours. The materials are suitably placed in an incubator at 42 ± 1°C and incubated on a shaking platform at 90 rpm for 24 ± 1 hours.

Use of Rappaport-Vassiliadis (RV) selective agents, which are believed to be the best of those in current use, requires reduction of the pH of the medium to 5.2 upon capsule release to achieve sufficiently selective conditions. In order to achieve this, both the capsule content formulation and the pre-enrichment medium have been manipulated. The key selective properties of RV are brought about by its high magnesium chloride content, malachite green, a pH of 5.2 and an incubation temperature of 41.5- 42.0°C. The other components of RV such as the phosphate buffer, sodium chloride and peptone are not required in the capsule formulation because they are preferably already present in the preferred pre-enrichment medium. Because of the buffered nature of the preferred pre-enrichment medium, capsule release of magnesium chloride does not naturally reduce the pH to a sufficient extent. For this reason, extra acid is preferably added to the capsule, conveniently in the form of malic acid. Malic acid is favoured because it is already frequently used in culture media to adjust pH. Without reducing the buffering capacity

of the pre-enrichment medium it would not be possible to fit all the required malic acid into a reasonable number of capsules, conveniently 6 capsules. It was discovered that a small portion of the magnesium chloride could be included in the pre-enrichment medium without damaging its recovery capability, thus providing more space in the capsules for malic acid. It was still necessary to reduce the buffering capacity of the pre-enrichment medium to limit the amount of malic acid to 0.8g per litre. Approximately 0.6g per litre of magnesium chloride was removed from the total test formulation without affecting selectivity.

Reducing the buffering capacity of the pre-enrichment medium is not a concern because of the shortened non-selective period for growth of organisms that may influence the pH of the medium. The medium still retains sufficient capacity to cope with the pH influence of the food material in the majority of samples. Foods with extreme pHs will require the broth pH to be adjusted to 7.0 by the addition of acid or alkali before the test is initiated.

Additional malachite green is preferably included in the capsule formulation when compared to the traditional RV formulation. This is to overcome any absorption of dye by the food material that is now present throughout the test period. The higher concentration of malachite green has been shown not to be toxic to dye-sensitive *Salmonella* strains.

As mentioned previously, when using RV selective agents an incubation temperature of 41.5 - 42.0°C is also critical to achieving sufficient selectivity to prevent overgrowth of competing organisms. Direct inoculation of samples into a broth at this temperature, however, is detrimental to the recovery of stressed cells. To overcome this it is convenient to use a gradual temperature increase brought about by controlling the temperature of the broth prior to incubation.

Shaking at 90 r.p.m. is required to achieve thorough mixing of the capsule contents once released. If the speed of shaking is too high then oxygen will be introduced with its associated toxic effects on stressed cells.

An optimised recovery period, followed by time-delayed delivery of selective agents, is equally applicable to the isolation of other important foodborne pathogens as it is to *Salmonella*. Oxidative stress is a problem in the recovery of all stressed foodborne pathogens so it is likely that a similar version of the modified pre-enrichment medium and OXYRASE supplement described above would be appropriate. In the case of *Campylobacter,* a medium containing blood and/or other protective agents maybe more suitable. A similar time delay before delivery of selective agents is also likely to be appropriate. In some circumstances, probably for *Campylobacter*, it may be necessary to incorporate some selective agents from the start of the test to overcome particularly acute forms of the Jameson Effect. Possible selective agents for *Campylobacter* include: (at time zero) cefperaxone, amphotericin, trimethoprim, and (delayed addition) polymixin and rifampicin.

For the isolation of *E. coli* O157 suitable selective agents for time-delayed delivery include bile salts and novobiocin and/or a combination of the following antibiotics: vancomycin, cefixime, cefsulodin and acriflavin. The medium could also include rhamnose or tellurite. All of these are currently used in selective media for the isolation and detection of *E. coli* O157.

For the isolation of *Listeria monocytogenes* suitable selective agents for delayed addition include: nalidixic acid, acriflavine and cyclohexamide. The addition of ferric ammonium citrate to the pre-enrichment medium may be appropriate to aid growth of *Listeria*.

The temperature of incubation may need to be varied for these other foodborne pathogens.

A chromogen may optionally be included in the test to give a colour reaction that could be measured at the end of the test to indicate the presence of absence of a particular organism.

In a preferred aspect, the present invention provides a method of enriching the population of *Salmonella* in a sample, comprising incubating the sample in a pre-enrichment medium comprising peptone and OXYRASE, with one or more timed-release capsules containing

Rappaport-Vassiliadis selective agents and malic acid, the capsules being arranged to release their contents approximately 5 hours after contact with aqueous medium, incubation being carried out for approximately 24 hours at a temperature rising to approximately 42°C.

In a further aspect, the present invention provides ingredients for use in enriching the population of a target microorganism in a sample, comprising a timed-release capsule containing one or more selective agents to favour growth of the target microorganism.

The ingredients preferably further comprise a supply of pre-enrichment medium, desirably comprising peptone with optional recovery agent, preferably OXYRASE. The OXYRASE is preferably in freeze-dried form.

The pre-enriched medium preferably includes a hydrogen donor in sufficient amount for optimum functioning of the enzymes contained in OXYRASE. Suitable hydrogen donors include lactic acid, succinic acid, formic acid, alpha glycerol phosphate and their salts. A millimolar concentration of the hydrogen donor is generally ample to remove all dissolved oxygen.

The invention will be further described, by way of illustration, in the following Examples and with reference to the accompanying figures in which:

Figure 1 is a simplified, schematic graph of cell numbers versus time;

Figure 2 is a schematic representation of a conventional method for detection of *Salmonella* in foods;

Figure 3 is a schematic sectional view of a delayed timed-release capsule;

Figure 4 is a chart of the release time of a number of capsules as shown in Figure 3; and

Figure 5 is a graph of temperature versus time, showing the temperature profile over 24

hours of a stomacher bag in an incubator set at 42°C.

Example 1

A product for use in enriching *Salmonella* in food samples comprises a pot containing 500g of dehydrated modified pre-enrichment medium (which is sufficient for about 100 tests), a vial of freeze-dried OXYRASE and a pack of 6 timed-release capsules containing selective agents. The vial contains about 50 units of OXYRASE (as measured according to the method described in the OXYRASE product literature) which is an appropriate amount for 1 test. The pot of enrichment broth will be sold separately. A kit containing enough OXYRASE and capsules for 20 tests, ie 20 vials of OXYRASE and 20 packs of 6 capsules, will be available separately.

The pre-enrichment medium is generally as described in GB 9721396.1 (with the addition of magnesium chloride, and with reduced buffering capacity), and is intended to be dissolved in distilled water in the amount of 4.275g of medium in 225 ml of water to produce a broth having the following composition:

	(g per litre)
Meat-based Peptone	10.0
Sodium chloride	5.0
Di-potassium hydrogen orthophosphate	1.0
Potassium di-hydrogen orthophosphate	0.4
Sodium succinate (anhydrous)	2.4
Magnesium chloride (anhydrous)	0.2

pH 7.2 ± 0.2

The peptone component of the pre-enrichment broth formulation is a meat based product comprising:

	% dry weight
meat meal	82.3
tryptone	13.9
yeast extract	2.5
disodium hydrogen orthophosphate	1.3

The riboflavin content of the pre-enrichment medium was measured by the technique described in Example 3 of GB 9721396.1, and was determined to be 0.045 mg/l. The hydrogen peroxide equivalent was measured by the technique described in Example 4 of GB 9721396.1, and was determined to be 0.021mM.

The freeze-dried OXYRASE enzyme was produced by freeze-drying in known manner OXYRASE enzyme obtained from Oxyrase, Inc., after removal of succinate and lactate if present.

The capsules are "O" size PULSINCAP capsules, as illustrated in Figure 3. The illustrated capsule comprises a generally cylindrical capsule body 10 of insoluble plastics containing a hard compacted cylindrical slug 12 of selective agents weighing 570 mg, the slug being 11mm in length and 6mm in diameter. The capsule is closed by a male hydrogel plug 14, 3.2mm long.

When the capsule is exposed to an aqueous medium, the hydrogel plug 14 swells slowly and disengages after a predetermined time interval, releasing the capsule contents. The capsules are designed to release the plug 5 hours ± ½ hour after exposure to an aqueous medium, as experiments on the lag phase of *Salmonella* have shown this is the optimum time. Figure 4 is a chart of the time of plug release for 72 such capsules incubated at 42°C with shaking at 90 rpm in the presence of a range of different foodstuffs, demonstrating plug release in this time window for the vast majority of capsules tested.

Each capsule contains the following selective agent formulation:

	grams
Magnesium chloride (anhydrous)	0.5325
Malachite green	0.0025
Malic acid	0.0333

6 capsules are used in a single test.

The selective agents are based on Rappaport-Vassiliadis selective agents, and require reduction of the pH of the medium to 5.2 upon capsule release to achieve sufficiently selective conditions. In order to achieve this it was necessary to manipulate both the capsule content formulation and the pre-enrichment medium. The key selective properties of RV are brought about by its high magnesium chloride content, malachite green, a pH of 5.2 and an incubation temperature of 41.5 - 42.0°C. The other components of RV such as the phosphate buffer, sodium chloride and peptone are not included in the capsule formulation because they are already present in the modified pre-enrichment medium. Because of the buffered nature of the modified pre-enrichment medium the capsule release of magnesium chloride does not reduce the pH to a sufficiently low level without adding extra acid, in the form of malic acid. Malic acid was chosen because it is already frequently used in culture media to adjust pH. Without reducing the buffering capacity of the pre-enrichment medium it would not be possible to fit all the required malic acid into the 6 capsules. It was discovered that a small portion of the magnesium chloride could be included in the pre-enrichment medium without damaging its recovery capability, thus providing more space in the capsules for malic acid. It was still necessary to reduce the buffering capacity of the pre-enrichment medium to limit the amount of malic acid to 0.8g per litre. Approximately 0.6g per litre of magnesium chloride was removed from the total test formulation without affecting selectivity.

Reducing the buffering capacity of the pre-enrichment medium was not a concern because of the shortened non-selective period for growth of organisms that may influence the pH of the medium. The medium still retained sufficient capacity to cope with the pH influence of the food material in the majority of samples. Foods with extreme pHs will

require the broth pH to be adjusted to 7.0 by the addition of acid or alkali before the test is initiated.

Additional malachite green was included in the capsule formulation when compared to the traditional RV formulation. This was to overcome any absorption of dye by the food material that is now present throughout the test period. The new concentration has been shown not to be toxic to dye sensitive *Salmonella* strains.

The succinate that is included in the modified pre-enrichment formulation acts as a hydrogen donor to the OXYRASE recovery agent. This is traditionally included in the OXYRASE formulation but we have found this affects the freeze drying process and for this reason it has been included in the primary medium. This does not affect the recovery ability of the medium and may indeed contribute in a positive manner by assisting in the buffering of the medium at pH 5.2 upon capsule release (pK_a of succinate = 5.11). Lactate was removed from the OXYRASE formulation for a similar reason but not included in the medium formulation as we have shown it not to be necessary in the function of OXYRASE in our test format.

The product is used in testing food samples for *Salmonella* as follows.

The *Salmonella* enrichment broth is prepared by suspending 4.275g of the pre-enrichment medium in 225 ml of distilled water and mixing to dissolve. The solution is distributed into containers and sterilised by autoclaving at 121°C for 15 minutes. The food sample and *Salmonella* enrichment broth are allowed to come up to room temperature. 25g of the food sample is placed into a stomacher bag, eg a wired Sewards stomacher closure bag. 225 mls of *Salmonella* enrichment broth is added to the stomacher bag. The freeze dried OXYRASE is rehydrated with 2mls of sterile distilled water and mixed gently to avoid frothing. The rehydrated OXYRASE is added to the stomacher bag in an amount to achieve a final concentration of 0.2 units per ml of pre-enrichment broth. The stomacher bag is placed into a stomacher machine and mixed for 30 seconds. Timed-release capsules are added to the stomacher bag. The food samples are placed into a 10-place stomacher rack, put on to a platform shaker in an air circulating incubator set at a temperature of

42°C and the shaker set at 90 rpm (1.9 cm diameter circular orbit). Figure 5 is a graph showing the temperature profile over 24 hours of a stomacher bag containing 225 ml pre-enrichment medium, 25 ml water (in place of a sample) and 6 capsules, placed in the centre of the bag rack of an otherwise empty shaker in an air circulating incubator at 42°C, and also shows the timing of bursting of the 6 capsules. After 24 hours incubation, the rack is taken out of the incubator. After this the pre-enrichment and enrichment steps are complete.

Further testing may be performed as desired, eg by subculturing on selective plates and performing confirmatory tests on any suspect *Salmonella* colonies, or by using a rapid technique of choice.

As mentioned previously, when using RV selective agents an incubation temperature of 41.5 - 42.0°C is critical to achieving sufficient selectivity to prevent overgrowth of competing organisms. Direct inoculation of samples into a broth at this temperature, however, is detrimental to the recovery of stressed cells. To overcome this we have devised a gradual temperature increase brought about by controlling the temperature of the broth prior to incubation, as described above.

Shaking at 90 r.p.m. is required to achieve thorough mixing of the capsule contents once released. If the speed of shaking is too high then oxygen will be introduced with its associated toxic effects on stressed cells.

Example 2

Comparative tests were carried out to compare results obtained using the product described in Example 1 with results obtained using the existing England and Wales PHLS standard method based on BS5763: Part 4, 1993, for detection of *Salmonella* in artificially contaminated food samples of various types.

Foods

The following five types of food samples were tested: cooked chicken, milk powder, chilled ready meal, soft cheese, ice cream.

Inoculation

Each of ten of the most common serotypes of *Salmonella* associated with food were inoculated into each of the food types, at inoculum levels of approximately < 10 cfu/25g sample. Test isolates were in most cases injured by heat treatment (51.5°C for 25 min) with the soft cheese samples being injured by low acid/high salt exposure (13½% NaCl, pH 4.5 at 4°C for 12 days). Uninoculated control samples were set up for each food. Each food/serotype combination was tested singly.

The samples were examined using both PHLS method and the method of the invention.

PHLS Standard Method

1. Aseptically chop the sample into small pieces and place 25g into a stomacher bag.

2. Add 225ml buffered peptone water (BPW) to the sample. Homogenise using a stomacher. Measure the pH. Incubate at 36°C ± 2°C for 18-24h.

3. a) Subculture 100µl of pre-enrichment culture into 10ml Rappaport-Vasiliadis soya peptone broth (RVS) medium and incubate at 42°C ± 1°C for 18-24h.

b) Subculture 1ml of pre-enrichment culture into 10ml selenite cystine broth and incubate at 36°C ± 2°C for 18-24h.

4. Subculture both enrichment broths onto xylose lysine desoxycholate (XLD) and modified brilliant green agar (BGA). Incubate at 37°C for 20-24h.

5. Examine plates for the presence of typical colonies. Select three suspected *Salmonella*

colonies from each of the selective agar plates and confirm using TSI and LSI slopes and serological tests (Poly O and Poly H). Any colonies found not to be *Salmonella* spp. should be identified using API 20E kits.

New Method

1. Aseptically chop the sample into small pieces and place 25g into a stomacher bag with wire closure.

2. Add 225ml of modified pre-enrichment broth to the sample and recovery agent (freeze dried supplement to be rehydrated in sterile water), and homogenise using a stomacher. Measure the pH. If the pH of the broth is less than 6.5 or greater than 7.2 the pH should be brought back to 7.0 by the addition of sterile 1M HCl or NaOH.

3. Ensure that the broth/food mixture is at ambient temperature (18-25°C), add six capsules containing the selective agents for delayed release. Fold down the top of the bag. Place bag in plastic stomacher bag rack and put on a shaking platform inside air circulating incubator at 42°C ± 1°C. Incubate on shaking platform at 90 rpm (1.9cm diameter circular orbit) for 24 ± 1 hour.

4. Carefully open the stomacher bag. Subculture using a 10μl plastic loop (avoiding food debris) onto XLD and BGA plates. Incubate plates at 37°C for 20-24h.

5. Examine plates for the presence of typical colonies. Select three suspected *Salmonella* colonies from each of the selective agar plates and confirm using TSI and LSI slopes and serological tests (Poly O and Poly H). Any colonies found not to be *Salmonella* spp should be identified using API 20E kits.

The growth of *Salmonella* on the selective agar plates from both methods was semi-quantified.

Results are summarised in the following table.

Sample Type	Number of serotypes	Number of positive results New Method	Number of positive results PHLS
Cooked Chicken	10	9	9
Milk powder	10	7	6
Chilled Ready Meal	10	9	9
Soft cheese	10	5	5
Ice cream	10	9	9

The results were uniformly good for all serotypes.

Example 3

Using the procedures described in Example 2, similar comparative tests were carried out on naturally contaminated food samples of various types.

Results are summarised in the following table:

Sample Type	Number of Samples	Number of positive results New Method	Number of positive results PHLS
Animal feed	80	15	15
Sausage	230	41	35
Poultry	120	54	42
Vegetables	60	0	1
Liquid raw egg	40	28	25

CLAIMS

1. A method of enriching the population of a target microorganism in a sample, comprising incubating the sample in a pre-enrichment medium, with one or more selective agents to favour growth of the target microorganism arranged for the release into the medium after a predetermined time delay.

2. A method according to claim 1, wherein enrichment is followed by identification of the target microorganism.

3. A method according to claim 1 or 2, wherein the pre-enrichment medium comprises peptone.

4. A method according to claim 1, 2 or 3, wherein the pre-enrichment medium comprises one or more recovery agents, preferably OXYRASE.

5. A method according to any one of the preceding claims, wherein the selective agents(s) are in one or more timed-release capsules.

6. A method according to claim 5, wherein each capsule comprises a water insoluble body with a hydrogel plug.

7. A method of enriching the population of *Salmonella* in a sample, comprising incubating the sample in a pre-enrichment medium comprising peptone and OXYRASE, with one or more timed-release capsules containing Rappaport-Vassiliadis selective agents and malic acid, the capsules being arranged to release their contents approximately 5 hours after contact with aqueous medium, incubation being carried out for approximately 24 hours at a temperature rising to approximately 42°C.

8. A method according to any one of the preceding claims, for testing samples of foodstuffs, beverages and environmental samples.

9. Ingredients for use in enriching the population of a target microorganism in a sample, comprising a timed-releasing capsule containing one or more selective agents to favour growth of the target microorganism.

10. Ingredients according to claim 9, further comprising a supply of pre-enrichment medium, preferably peptone, optionally with one or more recovery agents, desirably OXYRASE.

11. Ingredients according to claim 10, wherein the OXYRASE is in freeze-dried form.

12. Ingredients according to claim 10 or 11, wherein the pre-enrichment medium includes a hydrogen donor, preferably succinate.

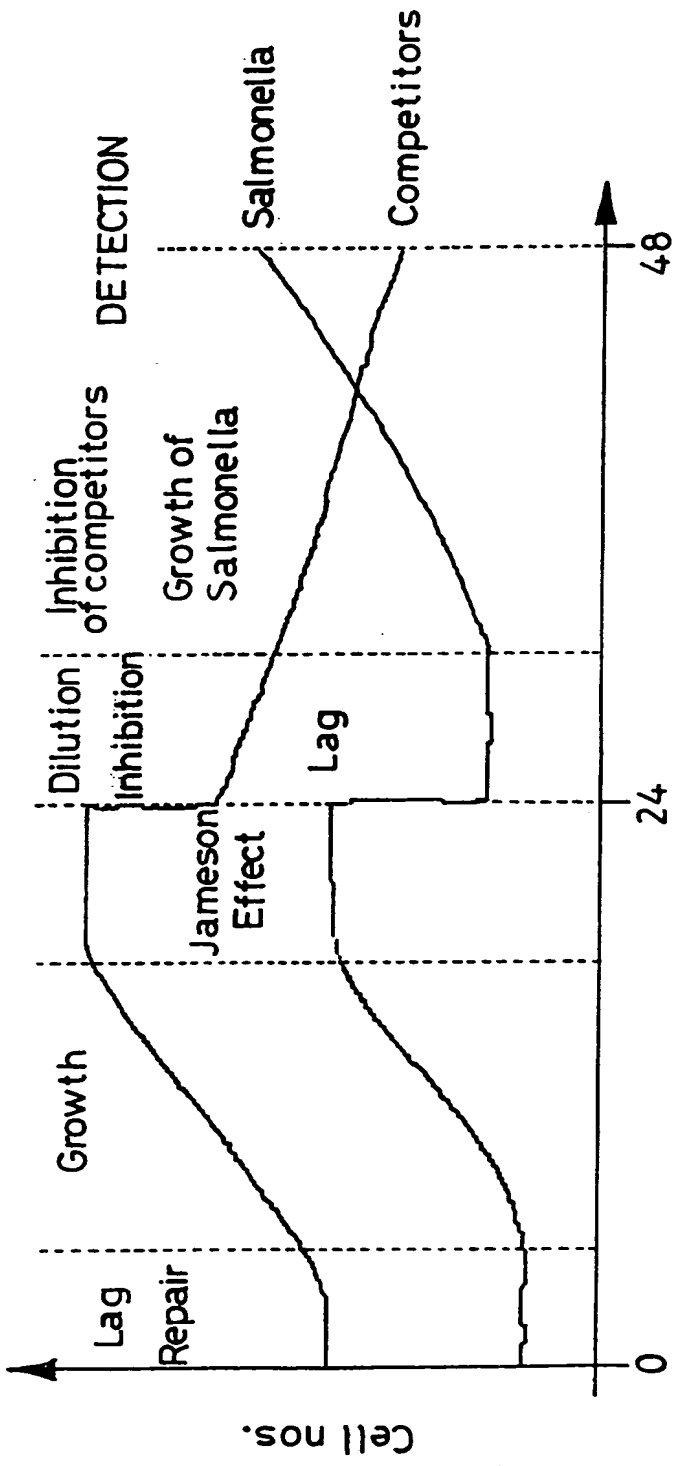

Fig. 1

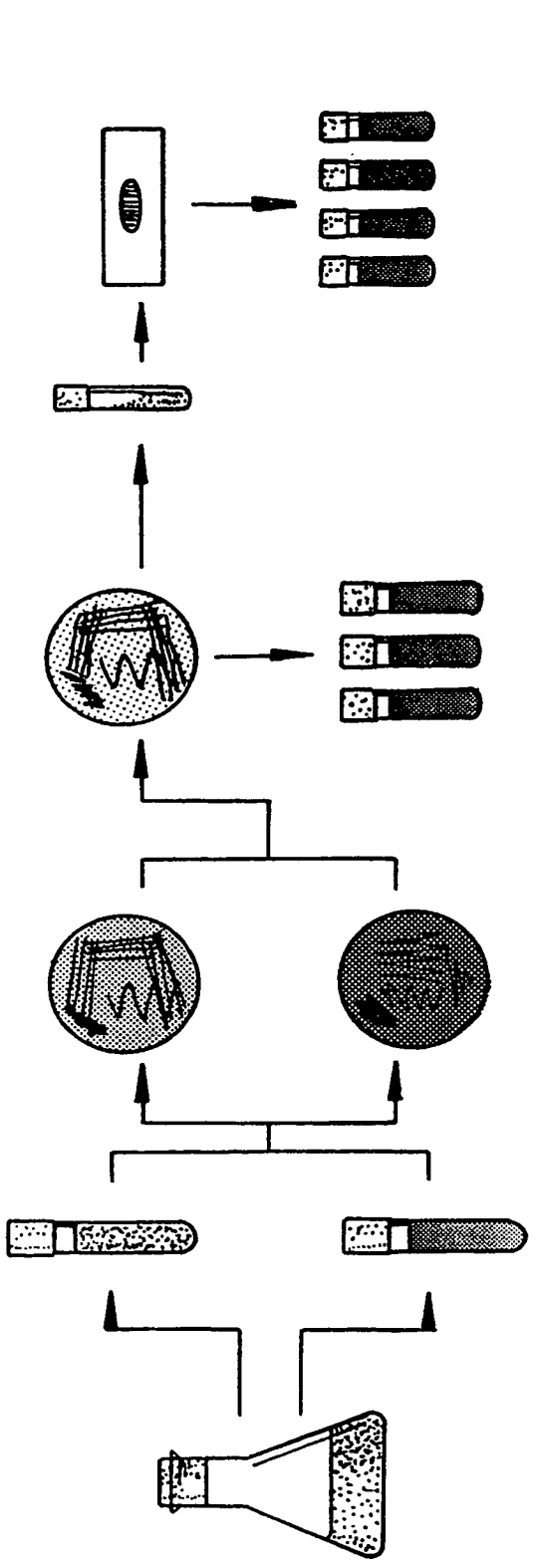

Fig. 2

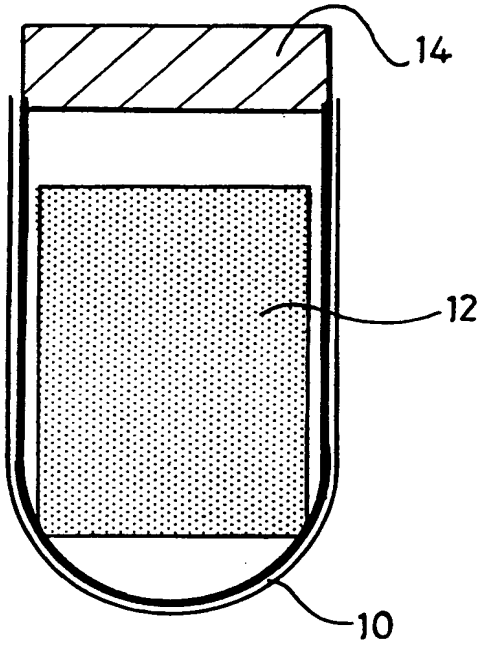

Fig. 3

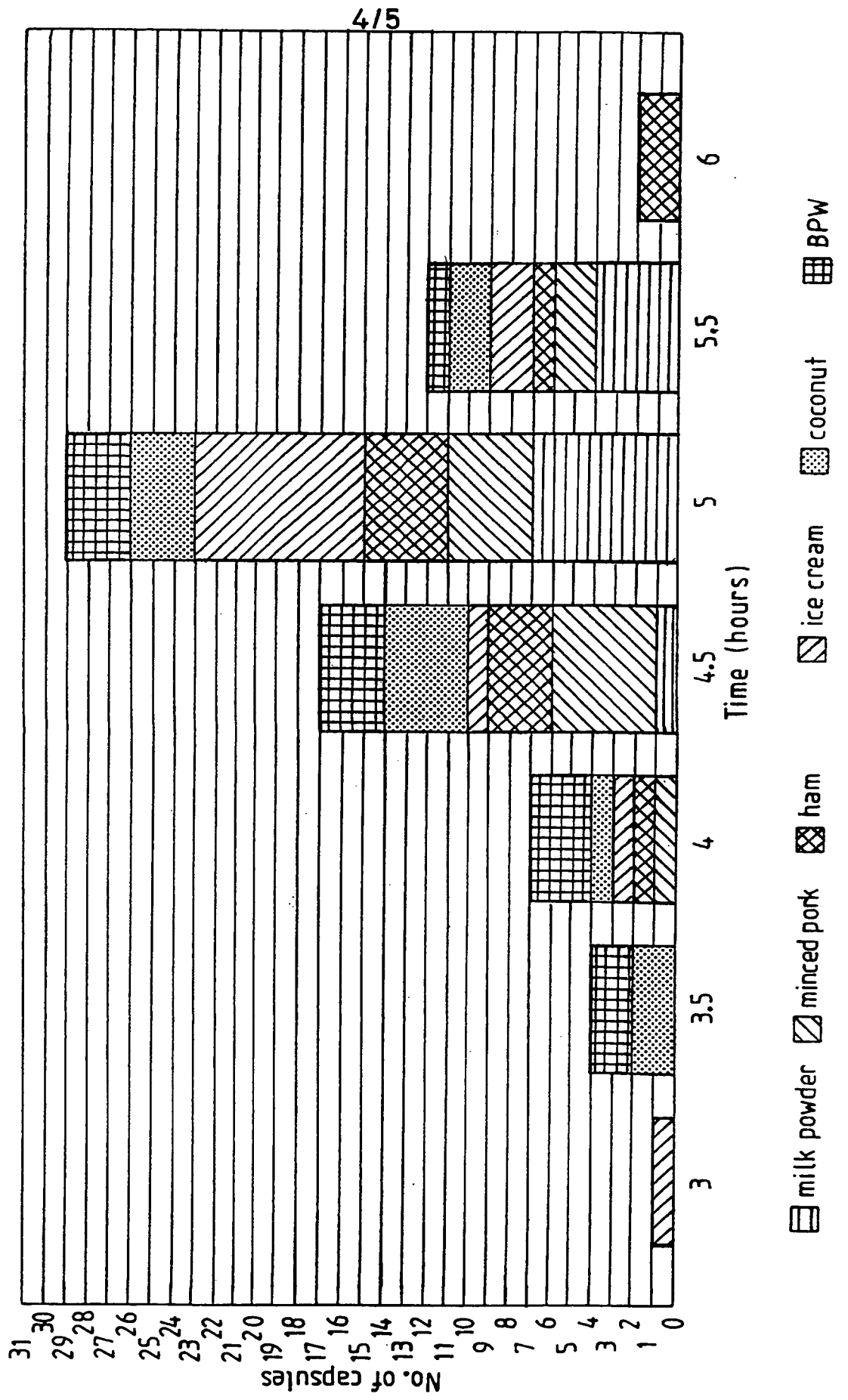

Fig. 4

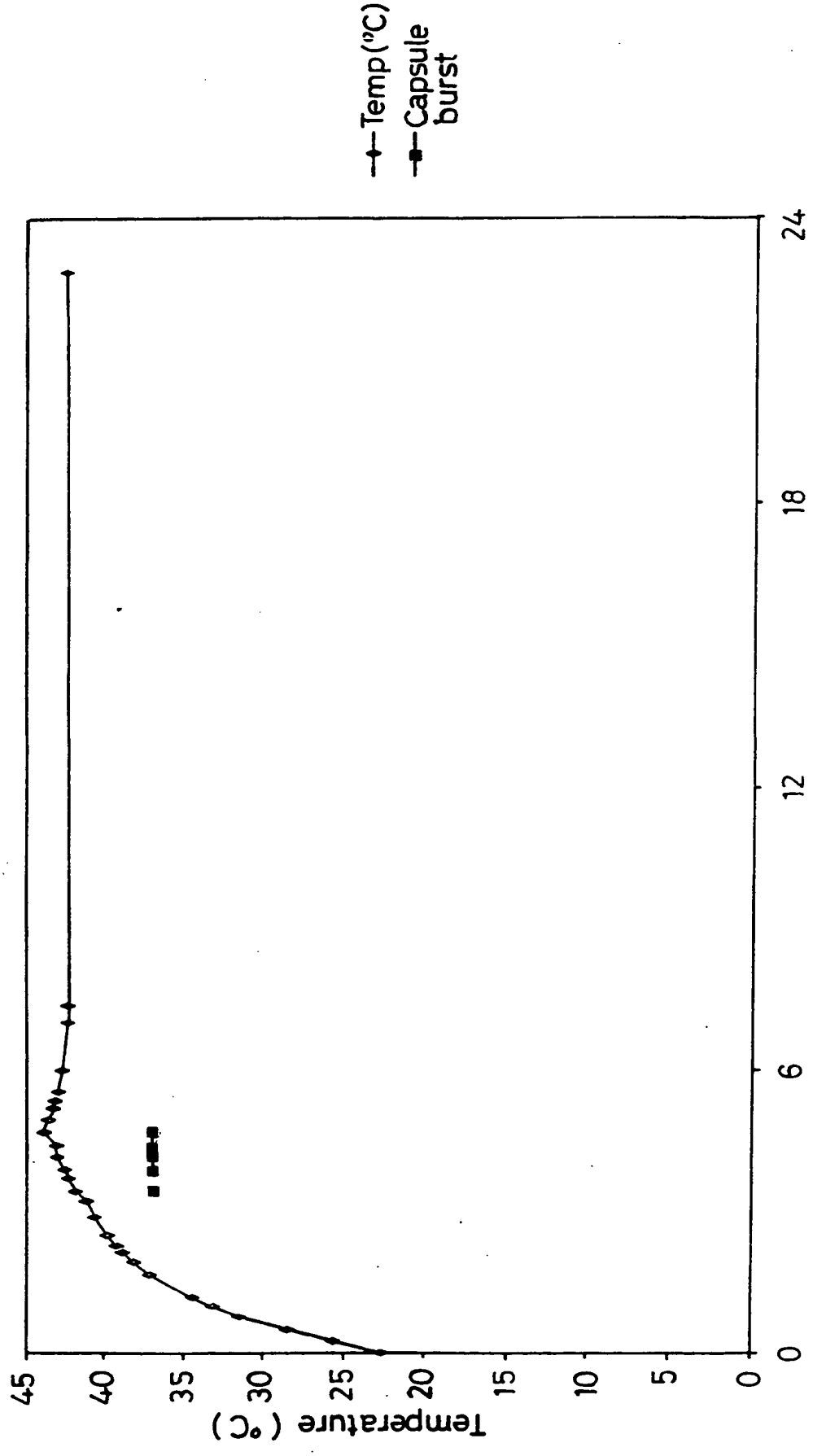

Fig. 5

ID: <WO___9902650A1_I_>

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PC 98/02016

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N1/38 C12Q1/04 C12N1/20 //C12Q1/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SVEUM W H ET AL: "Timed-release capsule method for the detection of Salmonella in foods and feeds" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 33, no. 3, 1977, pages 630-634, XP002069668 WASHINGTON US see the whole document ---	1,2,5, 8-10
A	WO 96 00794 A (VAMOS GYULA) 11 January 1996 see the whole document ---	1,3,7,8, 12
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 November 1998	12/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	De Kok, A

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INTERNATIONAL SEARCH REPORT

Inter[redacted]ication No
PCT/GB[redacted]02016

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIS F ET AL: "Cultural detection of Salmonella in raw milk. II. Our investigations" ARCHIV FÜR LEBENSMITTELHYGIENE, vol. 46, no. 3, 1995, pages 51-60, XP002069669 ALFELD DE see the whole document	1,2,7,8
A	WO 95 10263 A (R P SCHERER CORP) 20 April 1995 cited in the application see abstract	1,5,6
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[redacted]ion on patent family members

Interr [redacted]nal Application No
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